cells, other effector cells like NK cells are likely to exert GvL effects and may contribute to the favorable EFS of our patients.

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MEK INHIBITION RESULTS IN SELECTIVE IMMUNOSUPPRESSION OF HUMAN T CELLS AND DECREASES CYTOKINE PRODUCTION IN A MEMORY STAGE-DEPENDENT MANNER

Kim, T.K.[1,2], Valencia Gattas, M.[1], Benjamin, C.L.[1], Levy, R.B[1], Wieder, E.D.[1], Komanduri, K.V.[1,2] [1] University of Miami Sylvester Cancer Center, Miami, FL; [2] M.D. Anderson Cancer Center, Houston, TX

Although allogeneic stem cell transplantation is a treatment of choice for many hematologic malignancies, a key complication is graft-versus-host disease (GVHD). Although currently used immunosuppressive medications may successfully inhibit GVHD, none are selective enough to spare pathogen-specific T cells, leading to an increased risk of life-threatening infections (e.g., CMV reactivation). Recent studies suggest that naive and early memory T cells may be relatively more potent at inducing GVHD, whereas we and others have shown that most CMV-specific T cells exist as relatively differentiated cells. Therefore, we reasoned that differences in signaling could be used to selectively inhibit certain maturation stages of human T cells. Since the MAPK pathway has been demonstrated to be important for cytokine production in T cells, we measured phospho-Erk1/2 (p-Erk) in PMA:Ionomycin-treated or SEB-stimulated CD4+ and CD8 + T cell maturation subsets by flow cytometry. Naive (CD27 + CD45RA+) and early memory (M1, CD27 + CD45RA-) T cells expressed three-fold higher p-Erk than intermediate (M2, CD27-CD45RA-) and late memory (M3, CD27-CD45RA+) T cells ($P < 0.05$). To test the hypothesis that the effects of MEK inhibition depend on p-Erk expression, we stimulated T cells from healthy donors using allogeneic dendritic cells (DC) and assessed alloactivation in the presence or absence of the MEK inhibitor U0126. U0126-treated CD4+ and CD8 + T cells were 60-70% less activated than DMSO-treated cells, as assessed both by CD38 upregulation and CFSE dilution. Next we determined whether U0126 selectively suppresses cytokine production in a stage-dependent manner. We stimulated PBMC from healthy subjects (n = 7) with SEB in the presence of U0126 and measured IL-2, IFNγ and TNFα production in T cell maturation subsets. In CD4 + T cells, both IL-2 and TNFα production were significantly more suppressed in naive and early memory cells (Table 1). In CD8+ cells, we similarly saw reductions in IFNγ and TNFα production following MEK inhibition (Table 1). Preliminary experiments also suggest that U0126 only minimally suppresses of CMV pp65-specific T cells from healthy subjects (n = 3), although additional confirmation is needed. These data demonstrate that MEK inhibition preferentially inhibits naive and early memory cells critical for GVHD initiation while sparing late-memory CMV-specific cells, providing the basis for more selective clinical strategies for GVHD inhibition.

Mean percent reduction in cytokine production in response to U0126 (n = 7)

Cell	Cytokine	N	M1	M2	M3
CD4+	IL-2	79%	56%	50%	**
CD4+	TNFα	87%	62%	48%	**
CD8+	IFNγ	58%	30%	15%	0%
CD8+	TNFα	70%	55%	38%	5%

*all $P < 0.05$ for CD4: N vs. M1, M1 vs. M2, CD8: N vs. M1, M1 vs. M2, M2 vs. M3.

**M3 cells are not significantly present in CD4+ cells.

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STATINS INHIBIT THE ANTIGEN-PRESENTING FUNCTION OF HUMAN B CELLS

Shimabukuro-Vornhagen, A.[1,2], Zoghi, S.[2], Liebig, T.[2], von Bergwelt-Baildon, M.[1,2] [1] University Hospital of Cologne, Germany; [2] University Hospital of Cologne, Germany

Statins are lipid-lowering drugs that reduce cholesterol production by inhibiting HMG-CoA-reductase the rate-limiting enzyme of the mevalonate pathway. Besides their lipid-lowering effect statins possess potent immunomodulatory activity. Apart from T cells several other immune cells, including B cells, have been shown to be involved in the pathophysiology of graft-versus-host disease (GVHD). We conducted a expression analysis which showed that the genes of the mevalonate pathway were upregulated following activation of human B cells via CD40. Therefore we tested whether statins could inhibit the activation and antigen-presenting function of B cells as well as their ability to stimulate the proliferation of allogeneic T cells. Addition of simvastatin led to a dose-dependent inhibition of B cell activation and proliferation following stimulation via CD40. The upregulation of important costimulatory molecules such as CD80 and CD86 as well as MHC class II was significantly reduced when simvastatin was added to co-cultures of purified human B cells with CD40 ligand-expressing fibroblasts. As a consequence the proliferation of allogeneic T cells in response to simvastatin-treated CD40-activated B cells was significantly decreased compared to untreated controls. Addition of atorvastatin had similar effects on CD40-activated B cells. Surprisingly, the hydrophilic statin pravastatin had no significant effects on B cell activation and antigen-presenting function. Importantly, the doses of simvastatin that were required for inhibition of the antigen presenting function of B cells were at least one order of magnitude lower than those required to inhibit T cell activation and proliferation induced by anti-CD3/anti-CD28-coated beads. These results therefore suggest that inhibition of antigen-presenting cells is an important mechanism by which statins mediate their immunomodulatory effects. Furthermore, the observation that pravastatin had no significant effect on antigen-presenting function has important clinical implications and indicates that hydrophilic statins like simvastatin should be prefered in future clinical trials assessing the impact of statins on GVHD.

In summary, our findings demonstrate that statins are promising immunodulatory agents for the prevention and treatment of GVHD. This notion is supported by the results of recent clinial trials which found a reduction in acute and chronic GVHD in patients which were treated with statins.

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PRIMARY HUMAN ACUTE MYELOID LEUKEMIA STEM CELLS SHOW REDUCED SENSITIVITY TO NATURAL KILLER CELL-MEDIATED KILLING

Gill, S.[1], Majeti, R.[2], Bowler, M.[1], Negrin, R.S.[1] [1] Stanford University, Stanford, CA; [2] Stanford University, Palo Alto, CA

Introduction: Relapse after initial response to treatment of acute myeloid leukemia (AML) may be due to the persistence of leukemia stem cells (LSC), which show resistance to chemotherapy. Currently, the optimal approach to the targeting of LSC is unknown. In this study, we sought to investigate the potential graft-versus-leukemia effect of allogeneic natural killer (NK) cells on AML LSC using immunophenotypic and functional studies.

Methods: Samples were obtained from the peripheral blood of 11 newly diagnosed patients with AML, under an institutional review board-approved protocol. AML sub-populations were defined based on expression of CD34 and CD38. Initial analysis included cytofluorometric immunophenotyping of the NK receptor ligand molecules MICA/B, CD112, CD155, HLA-E, MHC class I, CD48, Fas, DR4 and DR5 in the different AML subpopulations. Of the 11 samples analysed, four contained an indentifiable distinct CD34 + CD38- population (defined immunophenotypically as LSC compared to the more mature population with the phenotype of CD34 + CD38+), and these were selected for functional studies. Subpopulations were separated using fluorescence-activated cell sorting and incubated with effectors (interleukin-2 stimulated purified allogeneic NK cells and the NK-92 cell line).

Results: Analysis of NK receptor ligands revealed non-significantly lower expression amongst the LSC population of the activating NKG2D ligands MICA/B (mean 10.9% +- 2.0 vs 34.7% +- 11.8, $p = 0.16$) as well as of the 2B4 ligand CD48 (mean 0.5% +- 0.2 vs 10.3% +- 4.9, $p = 0.15$). Using a panel of four different effectors, the putative LSC population and the non-LSC blast population were found to respond differentially to NK cell-mediated cytototoxicity, with caspase activation (as a measure of apoptosis) in the blast

population ranging up to 80% at an effector to target ratio of 40:1. The LSC population however was found to be uniformly less sensitive to NK cells than the non-LSC blast population, exhibiting apoptosis that was 45-85% of the more mature population ($p = 0.0002$ at 10:1 effector-to-target ratio; $p = 0.017$ at 40:1 effector-to-target ratio).

Conclusions: Immunophenotypically-defined AML stem cells show a reduced sensitivity to killing by allogeneic NK cells, a result that may be related to lower levels of expression of the activating ligands MICA/B and CD48. These results may have implications for the utility of natural killer cells in eradicating AML.

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ROLE OF INTERFERON-INDUCED PROCESSES IN LICHENOID SKIN INFLAMMATION IN CHRONIC GRAFT VERSUS HOST DISEASE

Hakim, F.T.[1], Imanguli, M.[1], Lafond, S.[1], Swaim, W.[3], Rehmann, N.[1], Memon, S.[1], Cowen, E.[2], Pavletic, S.[1], Gress, R.E.[1] [1] National Cancer Institute, NIH, Bethesda, MD; [2] National Cancer Institute, NIH, Bethesda, MD; [3] National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD

Chronic graft versus host disease (CGHD) remains a major non-relapse source of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (HSCT), but little is known of its pathogenesis. As part of an ongoing NCI CGVHD natural history protocol (04-C-0281), paired affected and unaffected skin biopsies were collected from patients with cutaneous CGVHD. Using multi-parameter fluorescent immunohistochemistry, we assessed the dense subepidermal infiltrate of mononuclear cells in skin biopsies with lichenoid histological findings in 9 patients. The primary T cell population in affected skin biopsies consisted of Th1/Tc1 cells expressing the transcription factor T-Bet. CD8 + T cells predominated in tissue from patients fewer than 9 months after HSCT, but were less than CD8-CD3+ cells in biopsies taken from patients more than one year after HSCT. Both CD68+ myeloid cells in the dermal infiltrate and epidermal keratinocytes expressed elevated levels of Interferon (IFN) inducible factors, including MxA and the chemokine MIG (CXCL9). Concomitantly, most T cells in the infiltrate expressed the T-Bet induced receptor CXCR3 that binds MIG. These findings are consistent with a pathogenic process in which IFN produced by keratinocytes and infiltrating myeloid elements could support the recruitment of CXCR3+ effector cells into the skin. Elevated levels of IFN-inducible factors are also evident systemically. When cells from normal donors were cultured with as little as 2 pg/ml of Type I IFN, mRNA expression of the IFN-inducible genes BAFF, MxA and IFI44 increased by 2 to 4 logs. Plasma levels of the IFN-inducible factors MIG and BAFF were elevated in these patients (MIG median 1813 pg/ml; range 125-7440; BAFF median 4512; range 1453-14907 pg/ml) compared with normal donors. These data, and comparable observations we have made in oral CGVHD (Imanguli, Blood, 2009), support the hypothesis that IFN-mediated processes contribute to epithelial tissue damage in CGVHD.

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STAT1 SIGNALING IS CRITICAL FOR ACTIVATION AND DIFFERENTIATION OF ALLOREACTIVE DONOR LYMPHOCYTES DURING INDUCTION OF ACUTE GVHD FOLLOWING FULLY MHC-MISMATCHED AND MHC-MATCHED MHA-MISMATCHED BONE MARROW TRANSPLANTATION (BMT)

H-H., Ma[1], Lu, C.[1], Ziegler, J.[1], Lentzsch, S.[2], Mapara, M.Y.[1] [1] University of Pittsburgh Cancer Institute, PA; [2] University of Pittsburgh Cancer Institute, PA

We have previously demonstrated that activation of STAT1 and STAT3 in GVHD target tissues and secondary lymphoid organs belong to the earliest events during induction of GVHD. Using STAT1-gene-deficient (STAT1KO) mice we tested the role of donor STAT1 during GVHD induction in fully MHC-mismatched (129Sv[H2b] to BALB/c [H2d]) and MHC-matched minor histocompatibility antigen (mHA)-mismatched BMT (129Sv[CD45.2, H2b] to B6 [H2b] or B6.SJL [CD45.1, H2b] following lethal irradiation. Using STAT1KO whole splenocytes we were able to show that lack of STAT1 significantly inhibited development of GVHD in both major and mHA mismatched recipients with significantly extended median survival times (MST), lower GVHD morbidity and less inflammation in GVHD target tissue samples. Protection against GVHD in recipients of STAT1KO splenocytes was associated with significant inhibition of T cell activation as determined by changes in CD25, CD69 and CD62L expression on donor CD4+ and CD8 + T cells on days +6 post-BMT. Lack of STAT1 in donor splenocytes resulted in a significantly attenuated and skewed systemic inflammatory response on day +6 post-BMT as demonstrated by significantly reduced IFN-g levels ($p < 0.05$), but significantly increased IL-4 ($p = 0.003$), IL-5 ($p = 0.007$) and IL-17 ($p = 0.03$) levels. *In vitro* studies demonstrated that STAT1KO CD8 + T cells produced much less IFN-γ upon combined engagement of TCR and costimulation, but that this decrease in IFN-γ secretion could be rescued if cells were simultaneously cultured under Th1 conditions (i.e. in the presence of IL-12 and anti-IL4 antibody). In contrast, lack of STAT1 completely inhibited the differentiation of naive CD4 + T cells to IFN-γ-producing cells upon TCR commitment and this capacity was also severely impaired under Th1 conditions. These findings were confirmed *in vivo* in the MHC-mismatched GVHD setting as we were able to observe a significantly reduced number of donor-derived CD4 + IFN-γ-producing cells in recipients of STAT1 KO ($p = 0.018$) compared to recipients of WT cells, while there was no difference with regard to IFN-γ expression in CD8 + T cells. Most importantly, we observed a significant expansion of CD4 + CD25 + FOXP3 + Treg cells in recipients of STAT1KO splenocytes in both major MHC-mismatched and MHC-matched mHA-mismatched setting. Currently, studies are ongoing to assess the role of STAT1 in the generation and function of Treg cells during GVHD.

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A LOWER FOXP3 + T-REG/CD4 + CELLS RATIO EARLY AFTER HSCT MAY IDENTIFY PATIENTS AT RISK FOR ALLO-REACTIVE DISEASE

de Pagter, P.J.A.[1], Lindemans, C.A.[1], Reubsaet, L.L.[3], Baarle, D.[1,2], Keukens, L.[1], Nanlohy, N.[1], Sanders, E.A.M.[1], Prakken, B.[3], Kleer, I.M.[3], Boelens, J.J.[1] [1] Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, Netherlands; [2] Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, Netherlands; [3] Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, Netherlands

Regulatory CD4 + FOXP3 + T-cells (FOXP3 + Treg) are suggested to play a role in control of allo-reactive disease after haematopoietic stem cell transplantation (HSCT). We analyzed FOXP3 + Treg in relation to allo-reactive disease after cord blood (u-CB), matched unrelated donor (MUD) or identical sibling (id-Sib) HSCT.

In this prospective cohort study, absolute CD4 + T-cell numbers and the ratio of FOXP3 + Treg of total CD4 + T-cells were analyzed every other week by direct whole blood Fluorescence-activated cell sorting analysis. Early allo-reactive disease (<100days) was defined as acute Graft-versus-Host-Disease and/or idiopathic pulmonary syndrome after HSCT.

A total of 32 pediatric patients were included. Following myeloablative conditioning, 7 (22%) patients received a MUD, 13 (41%) id-Sib and 12 (38%) u-CB HSCT. These three patient groups showed significant differences in the FOXP3 + Treg reconstitution pattern. The FOXP3 + Treg/CD4+ ratio was higher in the first 6 weeks after id-Sib-HSCT compared to u-CB or MUD HSCT ($p = 0.018$). In u-CB recipients, the FOXP3 + Treg reconstitution was relatively slow. 12 of the 32 patients developed allo-reactive disease (8 aGvHD and 4 IPS). Overall, FOXP3 + Treg/CD4+ ratios were lower in patients prior to the developed allo-reactive disease compared to patients without allo-reactive disease ($p = 0.010$).

Development of allo-reactive disease is preceded by a low ratio of FOXp3 + Treg/CD4 + early after HSCT. The FOXP3 + Treg/CD4 ratio early (6 weeks) after HSCT might be used to identify patients at risk for allo-reactive disease and to guide immunosuppressive or immuno-therapy. Influencing the ratio of FOXP3 + Treg/CD4+ in vivo may influence the incidence of allo-reactive disease and may make HSCT safer.